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Adipose cell differentiation: evidence for a two-step process in the polyamine-dependent Ob1754 clonal line

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A subclone of preadipocyte Ob17 cells has been isolated (Ob1754 clonal line). Confluent Ob1754 cells treated with an inhibitor of spermidine and spermine synthesis, methylglyoxal bis(guanylhydrazone), were totally dependent upon putrescine addition for the expression of glycerol-3-phosphate dehydrogenase which behaved as a late marker of adipose conversion. Under these conditions, the early expression of lipoprotein lipase during growth arrest remained unchanged. Studies at the mRNA level showed that the expression of unidentified pOb24 and pGH3 mRNAs, which was parallel to that of lipoprotein lipase, is independent of polyamine addition whereas the late emergence of glycerol-3-phosphate dehydrogenase mRNA was putrescine-dependent and co-ordinated with the expression of pAL422 mRNA encoding for a myelin-P_2 homologue [Bernlohr, Angus, Lane, Bolanowski & Kelly (1984) Proc. Natl. Acad. Sci. U.S.A. **81**, 5468–5472]. The appearance of lipoprotein lipase preceded DNA synthesis and post-confluent mitoses which were both putrescine-dependent and which took place before the appearance of glycerol-3-phosphate dehydrogenase. Thus the adipose conversion of Ob1754 cells involves the expression of at least two separate sets of markers which are differently regulated.

INTRODUCTION

The chronological events occurring during the process of adipose conversion have been extensively investigated in preadipocyte cell lines and cell strains (Ailhaud, 1982). The question whether there is a differential expression of enzyme markers of adipose conversion after confluence has been studied in 3T3-F442A cells with respect to nutrient supply or agents affecting the cyclic AMP content (Kuri-Harcuch *et al.*, 1978; Spiegelman & Green, 1980). The results led to the distinction between 'primary' and 'secondary' enzymes of adipose conversion (Green, 1978). On the other hand, when 3T3-F442A cells were exposed to a single culture condition throughout the post-confluent phase, it was shown that, during the time course of the change of specific mRNA levels, those encoding for glycerol-3-phosphate dehydrogenase and an homologue of myelin-P_2 protein of M_r 13000 (Bernlohr *et al.*, 1984) accumulate before that encoding for an homologue of serine proteases of M_r 28000 (Spiegelman *et al.*, 1983; Cook *et al.*, 1985).

The time course of the change of phenotypic markers during adipose conversion of Ob17 cells has shown the following: (i) lipoprotein lipase and monoacylglycerol lipase emerge at confluence before any triacylglycerol accumulation both in serum-supplemented (Murphy *et al.*, 1981; Vannier *et al.*, 1985*a*) and serum-free (Gaillard *et al.*, 1985) medium, (ii) the accumulation of these lipids occurs later and coincides with the acquisition of glycerol-3-phosphate dehydrogenase required for the synthesis of the glycerol backbone (Vannier *et al.*, 1985*b*), and (iii) the emergence of glycerol-3-phosphate dehydrogenase is strongly dependent upon insulin added chronically to culture media whereas that of lipoprotein lipase is independent of the presence of this hormone (Amri *et al.*, 1984). Parallel experiments on the time course of the adipose conversion of Ob17 cells have also shown the occurrence of post-confluent mitoses (Djian *et al.*, 1982). This limited proliferation, first described in 3T3-F442A cells (Pairault & Green, 1979), appears to precede the emergence of glycerol-3-phosphate dehydrogenase (Kuri-Harcuch & Marsch-Moreno, 1983; Russel *et al.*, 1983).

Taken together, these observations were in favour of the existence of an early and late step in the expression of the differentiation programme of preadipocyte cells. In the present paper a direct proof of two distinct steps has been obtained by isolating and using a subclone of Ob17 cells (Ob1754 clonal line) which is totally dependent upon polyamines for complete adipose conversion. The relationships between these steps and post-confluent mitoses have been precisely delineated.

EXPERIMENTAL

Cell lines

Subclones of Ob17 cells (Négrel *et al.*, 1978) were selected after suspension of Ob17 cells in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal bovine serum, 200 units of penicillin/ml, 50 μg of streptomycin/ml, 33 μM-biotin and 17 μM-pantothenate (defined as standard medium). After counting with a Coulter counter, the cell suspension was diluted with standard medium to 1 cell/4 ml and inoculated in 24-multiwell plates (16-mm diameter; 1 ml of cell suspension per well). The same medium was changed once a week. On the second week 34 nM-insulin and 2 nM-tri-iodothyronine were added. After 2–3 weeks, some clones were selected for high or very low frequency of lipid-filled cells after exposure to the differentiation medium, i.e. standard medium supplemented with

17 nM-insulin and 2 nM-tri-iodothyronine. Among the clones showing the highest proportion of lipid-filled cells were selected the subclones 1753 and 1771. From the clones showing no lipid-filled cells was selected the subclone 1754 which proved to be dependent upon polyamine addition for complete adipose conversion (see the Results section).

Cell culture

Cells were plated at 1000 cells/cm^2 in 35-mm diameter dishes and they were grown in standard medium. After confluence (5 days after seeding), this medium was replaced by the differentiation medium supplemented or not with methylglyoxal bis(guanylhydrazone) and a polyamine as indicated. Media were changed every other day.

Cell-free extracts and enzyme assays

At 18–24 h after the last medium change, cells were rinsed twice at 37 °C with phosphate-buffered saline, pH 7.4 (140 mM-NaCl, 3 mM-KCl, 8 mM-Na_2HPO_4 and 1.5 mM-KH_2PO_4). Cell homogenates (from two pooled dishes) were obtained by using a Potter–Elvehjem homogenizer (20 strokes) with 20 mM-Tris adjusted to pH 7.5 with HCl; they were used directly for glycerol-3-phosphate dehydrogenase assays and protein determinations. For lipoprotein lipase assays, samples of homogenates were adjusted to 150 mM-NaCl, 2.5 mM-sodium barbital, pH 7.4, 140 mM-mannitol, 0.9 mM-$CaCl_2$, 0.25 mM-$MgCl_2$, 0.5 M-glycerol and 0.2% Triton X-114 and incubated for 2 h at 4 °C. As described by Bordier (1981), the detergent was removed by heat-treatment of the solubilized homogenate for 10 min at 30 °C followed by sedimentation for 10 min at 12000 *g*, and the supernatant was used for enzyme assays. Under these conditions, the recovery of lipoprotein lipase was 98–100% whereas > 95% of the initial amount of detergent was removed. Lipoprotein lipase (EC 3.1.1.34) and glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) activities were determined as previously described (Négrel *et al.*, 1978; Murphy *et al.*, 1981) and are expressed in nmol of product formed/min per mg of protein (i.e. unesterified fatty acid and NAD^+, respectively). Inter-assay variability did not differ by more than 5% and variability among mean values from separate dishes never exceeded 5%. Protein was determined with bovine serum albumin (fraction V; Sigma) as a standard (Lowry *et al.*, 1951).

Fluorescence microscopy

Immunofluorescence microscopy studies of lipoprotein lipase in Ob1754 cells were performed as described extensively by Vannier *et al.* (1985*a*) by using a goat anti-(lipoprotein lipase)IgG fraction (Etienne *et al.*, 1985).

Incorporation of [^{3}H]thymidine into DNA and autoradiography

[^{3}H]Thymidine incorporation into DNA was performed on Ob1754 post-confluent cells in 35-mm diameter dishes. Cells were maintained in the presence of 2 ml of the differentiation medium containing 3 μM-[*methyl*-^{3}H]thymidine (150 μCi/μmol) for 48 h. The cells were then washed twice with phosphate-buffered saline, pH 7.4, treated for 10 min at 4 °C with 5% (w/v) trichloroacetic acid and washed twice successively with 70% (v/v) ethanol, 90% (v/v) ethanol and absolute ethanol. Fixed cells were the solubilized in 1 ml of 0.1 M-NaOH and counted by adding 10 ml of Aquasure (New England Nuclear). Under these conditions, the rate of incorporation was constant up to 48 h and the level of incorporation did not exceed 6% of the added radioactivity. For autoradiographs, post-confluent cells were maintained in the differentiation medium containing 6 μCi of [*methyl*-^{3}H]thymidine (no carrier added). After 48 h the cells were fixed as described above and covered in the dish with emulsion (Kodak NTB2) previously warmed for 30 min at 40 °C. The autoradiographs were exposed for 7–9 days and then processed (2 min with Kodak developer D19B, 10 s with 0.1% acetic acid and 10 min with Kodak LX24 fixative).

Polyamine determination

Cells were rinsed at 4 °C with 50 mM-Tris adjusted to pH 7.4 with HCl and containing 0.1 mM-EDTA. Cells from duplicate dishes were scraped in the same buffer and homogenized in a Potter–Elvehjem homogenizer (20 strokes). Aliquots of homogenate were either used directly for glyclerol-3-phosphate dehydrogenase assays or adjusted as described above for lipoprotein lipase determinations. The remaining homogenate was deproteinized by the addition of $HClO_4$ (0.2 M final concentration), incubated at 4 °C for 2 h, and precipitates removed by centrifugation. Subsequently, the method of polyamine determination described by Chen *et al.* (1982) was used with slight modifications: dansylation of polyamines was carried out at 20 °C in the dark for 3 h only and the separation by h.p.l.c. was made by using a linear gradient of acetonitrile/water/trifluoroacetic acid (from 65:35:0.05 to 95:5:0.05, by vol.). The retention times were 6, 13 and 19 min at a flow rate of 2 ml/min for putrescine, spermidine and spermine, respectively.

RNA isolation

Cell monolayers were lysed directly in the culture dish by adding 50 mM-Tris adjusted to pH 7.5 with HCl and containing 5 M-guanidinium monothiocyanate, 10 mM-EDTA and 0.8 M-mercaptoethanol. Total RNA was precipitated by addition of LiCl (3 M final concentration) for 15 h at 4 °C. The RNAs were collected by centrifugation at 12000 *g* for 1 h, suspended in 10 mM-Tris adjusted to pH 7.4 with HCl and containing 1 mM-EDTA and 0.1% SDS. RNAs were then extracted with phenol and chloroform and precipitated at −20 °C by adding NaCl (0.1 M final concentration) and 2 vol. of ethanol (Cathala *et al.*, 1983). Total RNA concentration was determined by reading A_{260}. Poly(A)-containing RNAs were isolated by oligo(dT)–cellulose chromatography (Pharmacia PL Biochemicals) as described by Aviv & Leder (1972).

Northern blot and hybridization conditions

Poly(A)-containing RNAs (6 μg) were treated at 65 °C for 5 min with 1.1 M-formaldehyde in 50% formamide/10 mM-sodium phosphate, pH 6.5. Samples were electrophoresed in a 1.2% agarose gel containing 1.1 M-formaldehyde, 10 mM-sodium phosphate, pH 6.5, and 2 mM-EDTA. RNAs were transferred to nitrocellulose by using 3 M-NaCl/0.3 M-sodium citrate (Thomas, 1980).

Prehybridizations were performed overnight at 42 °C with 50% (v/v) formamide, 0.75 M-NaCl, 75 mM-sodium citrate, 50 mM-sodium phosphate, pH 6.5, 0.1% SDS, 0.1% poly(vinyl pyrrolidone), 0.1% Ficoll and 100 μg of denatured salmon sperm DNA/ml. Hybridizations were performed for 48 h under the same conditions with denatured ^{32}P-labelled nick-translated DNA probes (Maniatis *et al.*, 1975, 1982). Filters were washed three times in 0.3 M-NaCl/0.03 mM-sodium citrate/0.1% SDS at 37 °C for 30 min followed by a single wash in 0.03 M-NaCl/0.003 M-sodium citrate/0.1% SDS at 65 °C for 2 h. They were then exposed to Kodak XAR5 film at −70 °C with intensifying screens.

Materials

[*methyl*-^{3}H]Thymidine, [α-^{32}P]dCTP, tri[9,10-^{3}H]oleoylglycerol and nick translation kit were purchased from Amersham International (Amersham, Bucks., U.K.). Culture media and foetal bovine serum were from Gibco (Cergy-Pontoise, France). Rhodamine-conjugated rabbit anti-(goat IgG) was a product of Cappel Laboratories (West Chester, PA, U.S.A.). Guanidinium monothiocyanate was obtained from Fluka (Buchs, Switzerland). Other compounds were purchased from Sigma (St. Louis, MO, U.S.A.) Plasmids pA21, pC8 and pAL422 were respectively cDNA clones complementary to the mRNAs encoding mouse β-actin, mouse glycerol-3-phosphate dehydrogenase (Kozak & Birkenmeier, 1983) and a homologue of myelin-P_2 protein (Bernlohr *et al.*, 1984). Plasmid pGH3 was a clone complementary to an mRNA of 5 kilobases encoding a liver protein of M_r 36000 induced in high-carbohydrate-fed rats (Pichard *et al.*, 1985). Plasmid pOb24 was isolated from a pBr322 Ob17 adipocyte cDNA library; this cDNA clone corresponds to a mRNA of 6 kilobases that is induced at least 100-fold during differentiation of Ob17 and 3T3-F442A cells (our unpublished work).

RESULTS

Effect of polyamine treatment on the activity levels of glycerol-3-phosphate dehydrogenase in Ob1771 and Ob1754 cells

As shown in Fig. 1(*a*), the emergence of glycerol-3-phosphate dehydrogenase activity in confluent Ob1771 cells did not require the presence of a polyamine. However, a significant rise in activity levels was observed in the presence of high concentrations of added putrescine. A different picture emerged when Ob1771 cells are exposed to methyglyoxal bis(guanylhydrazone), a competitive and reversible inhibitor of *S*-adenosylmethionine decarboxylase. This key enzyme controls the concentration of decarboxylated *S*-adenosylmethionine which is normally very low and rate-limiting in the biosynthesis of spermidine and spermine. Moreover this enzyme is activated by putrescine in mammalian cells (Tabor & Tabor, 1976). A complete loss of cell viability made it impossible to perform experiments in the presence of methylglyoxal bis(guanylhydrazone) alone, whereas addition of 10 μM-putrescine or 0.3 μM-spermidine restored complete cell viability. In drug-treated Ob1771 cells, chronic exposure to concentrations of putrescine above 30 μM led to an increase in the glycerol-3-phosphate dehydrogenase activity, the maximal effect being observed at 100 μM (Fig. 1*a*). Spermidine at low concentrations (0.3–30 μM) could substitute for putrescine, whereas spermine (0.3–30 μM) was cytotoxic and prevented any significant conclusion being drawn (results not shown). Parallel observations reported in Fig. 1(*b*) have been made with confluent Ob1754 cells. In this case, however, the cells could not express the glycerol-3-phosphate dehydrogenase activity unless putrescine was added to the differentiation medium in the presence of methylglyoxal bis(guanylhydrazone). Spermidine from 0.3 to 100 μM was ineffective in replacing putrescine; the addition of 0.5 mM-aminoguanidine to

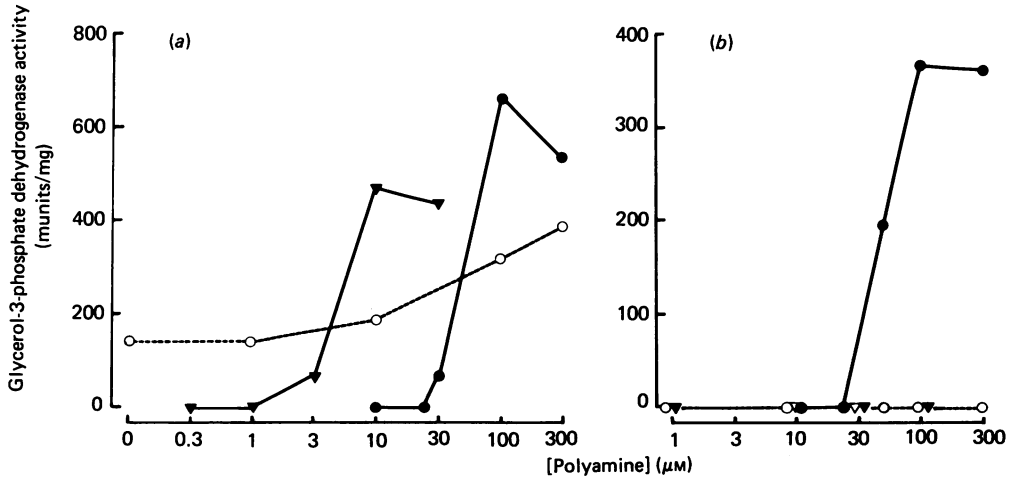

Fig. 1. Effects of polyamine treatment on the expression of glycerol-3-phosphate dehydrogenase in Ob1771 cells and Ob1754 cells

Ob1771 (*a*) and Ob1754 cells (*b*) were grown in standard medium. At confluence, the medium was supplemented with 17 nM-insulin, 2 nM-tri-iodothyronine and various concentrations of putrescine (○, ●) or spermidine (▽, ▼) in the absence (○, ▽) or presence (●, ▼) of 10 μM-methylglyoxal bis(guanylhydrazone). The glycerol-3-phosphate dehydrogenase activities were determined at 22 days after confluence. The curves shown are representative of experiments performed with at least three independent series of cells; the means of the maximal glycerol-3-phosphate dehydrogenase specific activities were: in (*a*), 480 ± 50 (with spermidine) and 693 ± 70 (with putrescine); in (*b*), 374 ± 40 (with putrescine).

Table 1. Polyamine concentrations and the activities of enzyme markers in confluent Ob1771 cells

Confluent Ob1771 cells were maintained for 4–9 days in the differentiation medium with the following supplementation: A, no addition; B, 20 μM-putrescine and 10 μM-methylglyoxal bis(guanylhydrazone); C, 100 μM-putrescine and 10 μM-methyglyoxal bis(guanylhydrazone). The intracellular polyamine concentrations were determined 4 days after confluence, 30 min after a last medium change (maximal levels were reached between day 3 and day 7). The enzyme activities were determined at day 9 post-confluence. The values reported (means ± S.D.) were obtained on six independent series of cells.

Culture condition	Glycerol-3-phosphate dehydrogenase (munits/mg of protein)	Lipoprotein lipase (munits/mg of protein	Intracellular polyamine concentrations (nmol/mg of protein)		
			Putrescine	Spermidine	Spermine
A	732±80	8.8±1.7	1.17±0.09	9.47±0.43	1.70±0.13
B	16.5± 6	7.7±1.7	11.21±1.49	4.26±0.32	0.45±0.02
C	1521±120	8.4±2	6.76±0.48	10.81±0.29	1.17±0.68

prevent oxidation by the serum made no improvement (Bethell & Pegg, 1981). The relationships between the polyamine concentrations and the activities of glycerol-3-phosphate dehydrogenase and lipoprotein lipase (see below) were examined in confluent Ob1771 cells. It should be noted in this respect that changes in both activities are directly correlated with changes in enzyme concentration (Spiegelman & Green, 1980; Vannier *et al.*, 1982). Table 1 shows a large decrease in the concentrations of spermidine and spermine, and an accumulation of putrescine, when Ob1771 cells were exposed to 10 μM-methylglyoxal bis(guanylhydrazone) and 20 μM-putrescine. Increasing the latter concentration to 100 μM-putrescine led to a significant rise in the intracellular concentrations of spermidine and spermine which attained 114% and 68% of the values for the untreated cells, respectively. Under these conditions the glycerol-3-phosphate dehydrogenase activity reached 207% of the control value. In the following experiments use has been made of Ob1754 cells since they are unable, in contrast to Ob1771 cells, to express glycerol-3-phosphate dehydrogenase in the differentiation medium alone while they are able under these conditions to express lipoprotein lipase (see below).

Kinetics of appearance of lipoprotein lipase and glycerol-3-phosphate dehydrogenase in Ob1754 cells

When confluent Ob1754 cells were treated with methylglyoxal bis(guanylhydrazone) and putrescine, a lag of at least 5 days was observed between the early emergence of lipoprotein lipase and the later emergence of glycerol-3-phosphate dehydrogenase (both enzymes were undetectable in exponentially-growing cells). When no treatment is given to Ob1754 cells, the emergence of lipoprotein lipase remained unchanged whereas that of glycerol-3-phosphate dehydrogenase was completely prevented (Fig. 2).

These results are in agreement with those obtained on Ob1771 cells (Table 1) which show that the activity of lipoprotein lipase is independent of the intracellular levels of spermidine and spermine. These results are also in agreement with micrographs of Ob1754 cells shown in Fig. 3: the immunofluorescence staining of lipoprotein lipase-containing cells indicates a very similar frequency (60–80%) of positive cells in treated (Fig. 3*e*) or untreated (Fig. 3*b*) cells. This frequency is within the range of that previously observed in Ob17 cells (Vannier *et al.*, 1982) and does not increase with time in culture. On the other hand, no lipid-filled cells were observed in untreated Ob1754 cells (Fig. 3*a*) compared with treated cells (Fig. 3*d*). This observation is in agreement with experiments using isopycnic centrifugation which showed that triacylglycerol accumulation occurred only in glycerol-3-phosphate dehydrogenase-containing cells (Vannier *et al.*, 1985*b*).

DNA synthesis and proliferation of confluent Ob1754 cells

Treatment of Ob1754 cells with methylglyoxal bis(guanylhydrazone) and putrescine was required for a wave of DNA synthesis to occur (Fig. 4). The cessation of this treatment led to a rapid decrease in DNA

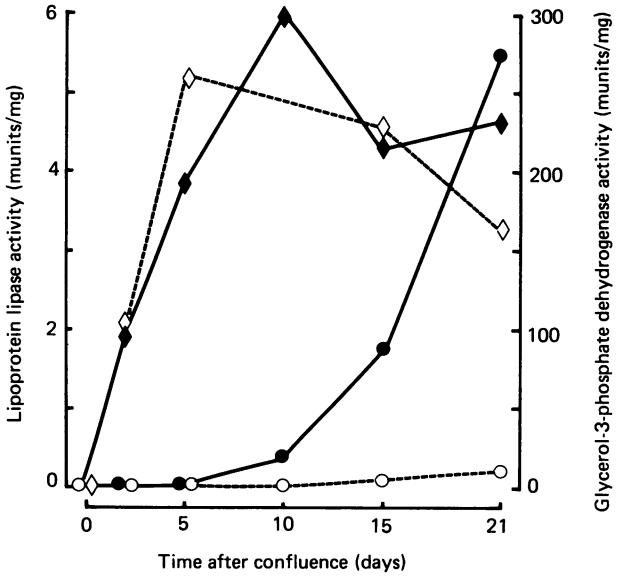

Fig. 2. Kinetics of appearance of lipoprotein lipase and glycerol-3-phosphate dehydrogenase activities in the presence of putrescine and methylglyoxal bis(guanylhydrazone)

Confluent Ob1754 cells were maintained in the presence of the differentiation medium (see the Experimental section) supplemented (●, ◆) or not (○, ◇) after confluence with a mixture of 100 μM-putrescine and 10 μM-methylglyoxal bis(guanylhydrazone). The lipoprotein lipase (◇, ◆) and glycerol-3-phosphate dehydrogenase (○, ●) activites were determined at the indicated times. Similar curves were obtained with three independent series of cells.

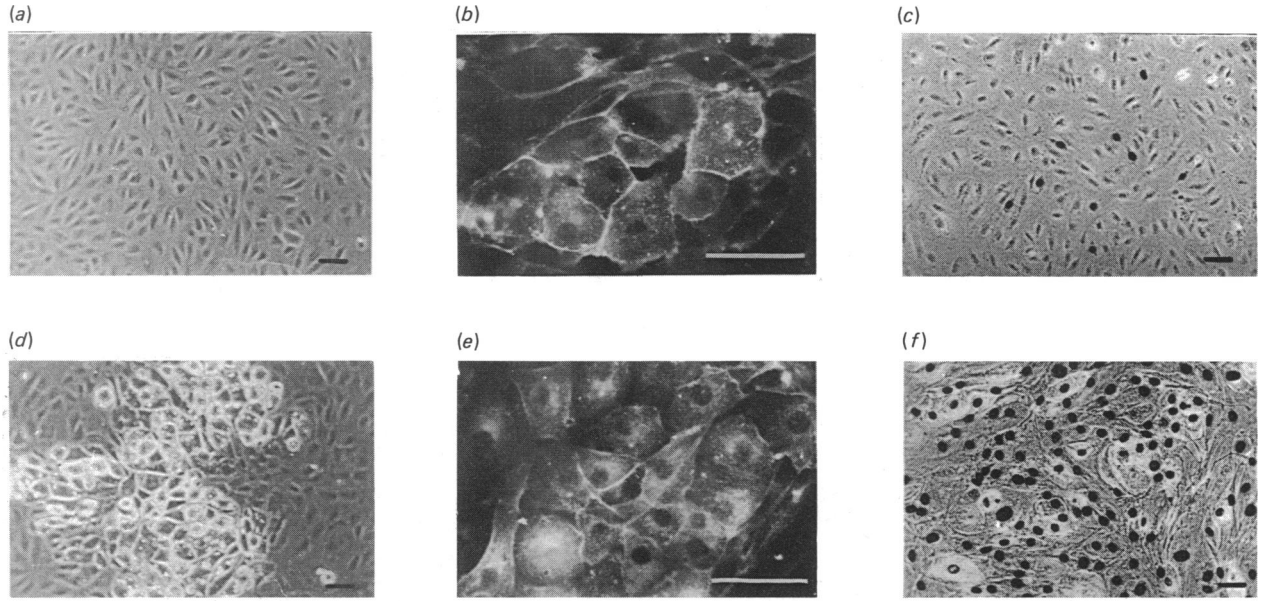

Fig. 3. Lipoprotein lipase-containing cells and DNA-synthesizing cells in the absence or presence of putrescine and methylglyoxal bis(guanylhydrazone)

Confluent Ob1754 cells were maintained after confluence in the differentiation medium supplemented (*d–f*) or not (*a–c*) with 100 μM-putrescine and 10 μM-methylglyloxal bis(guanylhydrazone). (*a*) and (*d*), Phase contrast of 18-day post-confluent cells; (*b*) and (*e*), immunofluorescence staining of lipoprotein lipase of 14-day post-confluent cells; (*c*) and (*f*), autoradiographs after a 48 h exposure to [^{3}H]thymidine between day 11 and day 13 after confluence. The bar is equivalent to 50 μm.

synthesis, indicating that the continuous presence of this mitogenic stimulus was needed. The wave of DNA synthesis lagged behind the full emergence of lipoprotein lipase but preceded that of glycerol-3-phosphate dehydrogenase. DNA synthesis has been visualized by the autoradiography experiments shown in Fig. 3. As expected, cell labelling was almost absent in unstimulated cells (Fig. 3*c*) compared with stimulated cells (Fig. 3*f*), in which the labelling index was estimated to be 50–60%. In agreement with the results on DNA synthesis, the determination of the cell number at day 22 showed a 1.2-fold increase for unstimulated cells and above a 2-fold increase for unstimulated cells. Taken together, the results of Figs. 2–4 would indicate that lipoprotein lipase is expressed early in growth-arrested cells. Provided they are exposed to an adequate mitogenic stimulus, lipoprotein lipase-containing cells then become able to synthesize DNA and to divide. The acquisition of glycerol-3-phosphate dehydrogenase clearly takes place after the main bulk of DNA synthesis and is not observed in the absence of DNA synthesis and mitoses.

Relationships in Ob1754 cells between the exposure time to methylglyoxal bis(guanylhydrazone) and putrescine and the expression of glycerol-3-phosphate dehydrogenase

Fig. 5 shows experiments in which confluent Ob1754 cells were exposed for different times (up to 22 days) to methylglyoxal bis(guanylhydrazone) and putrescine, the activities of glycerol-3-phosphate dehydrogenase being determined 22 days after confluence. This enzyme activity was increased when the exposure time was increased. Thus it appeared, as for DNA synthesis (Fig. 4), that a continuous exposure was required for a full expression. Secondly, however, it appeared that exposure from day 8 to day 22 after confluence was inefficient for the enzyme expression; in other words there was a critical period between confluence and day 8, at a time where growth was arrested and lipoprotein lipase was expressed, during which the cells also required the presence of methylglyoxal bis(guanylhydrazone) and putrescine for the expression of glycerol-3-phosphate dehydrogenase which took place approximately 1 week later (Fig. 2).

Northern blot analysis of poly(A)-containing mRNAs from unstimulated and stimulated Ob1754 cells

To test whether the expression of glycerol-3-phosphate dehydrogenase is correlated with the expression of its corresponding mRNA, Northern blot analyses have been performed. These analyses also included different mRNAs expressed during adipose conversion, including the mRNA encoding in 3T3-F442A cells for a protein of M_r 13000 which is co-ordinately expressed with the glycerol-3-phosphate dehydrogenase mRNA (Spiegelman *et al.*, 1983) (detected in the present study with pAL422 and pC8 cDNA probes, respectively). Both mRNAs were also co-ordinately expressed in late confluent Ob1771 cells; in addition two mRNA of 6 and 5 kilobases (detected with pOb24 and pGH3 cDNA probes respectively) appeared simultaneously with lipoprotein lipase in early confluent cells (our unpublished work). As shown in Fig. 6, exponentially-growing Ob1754 cells contained a very small amount of pOb24 mRNA, detectable only after long-term exposure of the films, and no pGH3, pAL422 and pC8 mRNAs (lane *a*). In the absence of methylglyoxal bis(guanylhydrazone) and putrescine, confluent Ob1754 cells accumulated pGH3 and pOb24 mRNAs whereas pAL422 and pC8 mRNAs remained undetectable (lane *b*). However, when these cells were exposed to the mitogenic stimulus, the mRNAs encoding

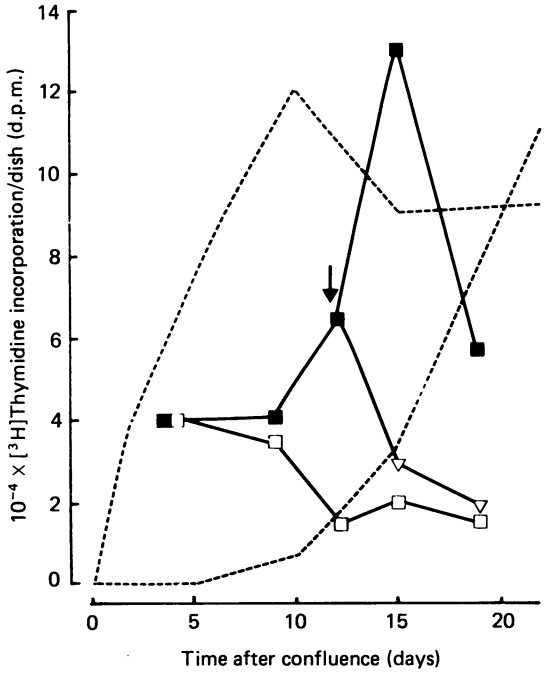

Fig. 4. DNA synthesis in the presence of putrescine and methylglyoxal bis(guanylhydrazone)

Confluent Ob1754 cells (from the series used in Fig. 2) were chronically exposed after confluence to the differentiation medium supplemented (■) or not (□) for 19 days with 100 μM-putrescine and 10 μM-methylglyoxal bis(guanylhydrazone). At day 12 (indicated by arrow), this treatment was stopped in one series of cells (▽). DNA synthesis was determined by [^{3}H]thymidine incorporation for 48 h into trichloroacetic acid-precipitable material. The broken lines are taken from Fig. 2 and represent lipoprotein lipase and glycerol-3-phosphate dehydrogenase activities in treated cells.

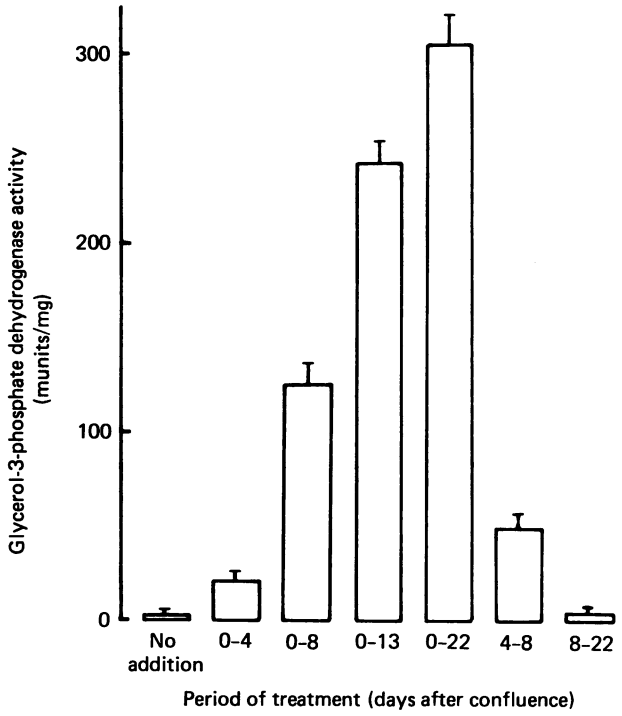

Fig. 5. Effect of exposure time to putrescine and methylglyoxal bis(guanylhydrazone) on the activity levels of glycerol-3-phosphate dehydrogenase in confluent Ob1754 cells

Ob1754 cells were maintained since confluence (defined as day 0) in the differentiation medium alone (no addition) or supplemented with 100 μM-putrescine and 10 μM-methylglyoxal bis(guanylhydrazone) for the time indicated. Under each condition, the glycerol-3-phosphate dehydrogenase activity was determined at 22 days after confluence. The activity values are reported as means ± ranges for two independent experiments.

for glycerol-3-phosphate dehydrogenase and myelin P_2-like protein appeared (lane *c*). The concentration of mRNA for actin did not differ significantly in any of the conditions used.

DISCUSSION

In the present work two subclones of Ob17 cells (Négrel *et al.*, 1978), the Ob1771 and mainly the Ob1754 clonal line, have been used to investigate the relationships between the expression of early and late markers of adipose conversion. Ob1754 cells have been characterized and proved to be totally dependent upon putrescine addition for the late emergence of glycerol-3-phosphate dehydrogenase provided that methylglyoxal bis(guanylhydrazone) was present. Similar observations have been made on Ob1771 cells. Methylglyoxal bis(guanylhydrazone) has been reported in many cell systems and tissues to increase the activity of *S*-adenosylmethionine decarboxylase indirectly by increasing the putrescine concentration and directly by increasing the enzyme amount (Shirahata & Pegg, 1985). Since it is known that the production of decarboxylated *S*-adenosylmethionine is rate-limiting for the synthesis of spermidine and since low concentrations of spermidine can replace high concentrations of putrescine in the case of Ob1771 cells (Fig. 1*a*), it is likely that the effect of methylglyoxal bis(guanylhydrazone) in these cells is to increase the activity of *S*-adenosylmethionine decarboxylase, leading in turn after putrescine addition to an increase in decarboxylated *S*-adenosylmethionine and spermidine concentrations. It is also likely that in Ob1754 cells an increase in spermidine concentrations above a critical level might play a similar role, since the spermidine content of unstimulated cells is below that of Ob1771 cells treated with 10 μM-methylglyoxal bis(guanylhydrazone) and 20 μM-putrescine (2.7 versus 4.26 nmol/mg of protein). However, the inability of spermidine to substitute for putrescine in Ob1754 cells (Fig. 1*b*) remains unclear. It should be recalled that a complex relationship seems to exist between polyamine metabolism and cellular differentiation, since a reduction in putrescine or spermidine concentrations and the extent of differentiation is observed in embryonal carcinoma cell lines (Schlinder *et al.*, 1983; Kelly *et al.*, 1985) and neuroblastoma cells (Chen *et al.*, 1982). By contrast, a potent increase in spermidine concentrations is correlated with the adipose conversion of 3T3-L1 preadipocyte cells (Bethell & Pegg, 1981) and L6 myoblast cells (Erwin *et al.*, 1983). The similar responses observed in 3T3-L1, Ob1771 and L6 clonal lines would suggest some relationship between the extent of differentiation and the

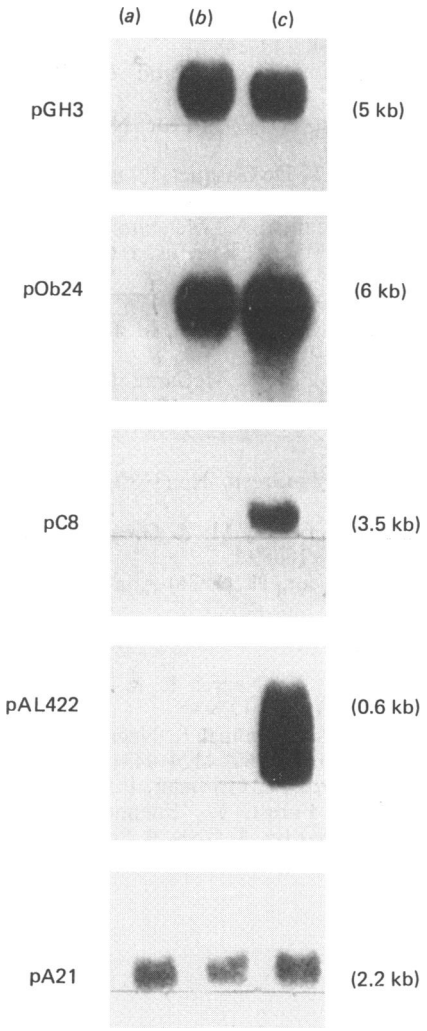

Fig. 6. Northern blot analysis of poly(A)-containing RNAs

Confluent Ob1754 cells were maintained under the same conditions as used in Figs. 2–5. Poly(A)-containing RNAs were prepared from (*a*) exponentially-growing cells, (*b*) 18-day post-confluent cells in the absence of any treatment, and (*c*) the same cells as in (*b*) but chronically treated with 100 μM-putrescine and 10 μM-methylglyoxal bis(guanylhydrazone). The different cDNA probes used (left) and the length of the corresponding mRNAs in kilobases (kb) (right) are indicated.

rise in spermidine concentrations which might be unique to cells of mesodermal origin.

The use of Ob1754 cells has allowed us to show that the emergence of glycerol-3-phosphate dehydrogenase does not take place in lipoprotein lipase-containing cells unless they are exposed to an appropriate stimulus [methylglyoxal bis(guanylhydrazone) plus putrescine]. The absence of a tight coupling between the expression of lipoprotein lipase and that of glycerol-3-phosphate dehydrogenase is in agreement with recent studies on Ob17 and Ob1771 cells which have shown that, in contrast to that of the second enzyme, the emergence of the first enzyme is independent of insulin, tri-iodothyronine and growth hormone (Amri *et al.*, 1984; Doglio *et al.*, 1986; our unpublished work). Additional studies at the mRNA level show the existence of at least two separate events in the differentiation program of Ob1754 cells: the expression of pOb24 and pGH3 mRNAs, which is co-ordinated with the early emergence of lipoprotein lipase, is indepedent of putrescine addition, whereas the simultaneous and late expression of pAL422 and glycerol-3-phosphate dehydrogenase mRNAs is dependent upon the presence of this polyamine.

The present study has also allowed us to gain some additional insight into the relationships between the expression of enzyme markers and post-confluent mitoses. Previous studies (Djian *et al.*, 1982) had shown that post-confluent, dividing cells were those which ultimately became adipose cells. The present results clearly show that post-confluent mitoses take place after the emergence of lipoprotein lipase and before that of glycerol-3-phosphate dehydrogenase (Figs. 2 and 4).

In light of the results reported above, the sequence of events taking place *in vivo* during the development of adipose tissue are better understood if one recalls that (i) lipoprotein lipase-containing cells are present in the stromal–vascular fraction of rat adipose tissue before any significant triacylglycerol accumulation (Hietanen & Greenwood, 1977; Pequinot-Planche *et al.*, 1977); (ii) after pulse-labelling with [^{3}H]thymidine, the labelling indices in rat subcutaneous fat organs are highest in partly differentiated cells containing no lipid droplets (Pilgrim, 1971) and (iii) the decrease in the labelling index of glycerol-3-phosphate dehydrogenase-negative cells precedes immediately the rise in the activity of this enzyme detected subsequently in all triacylglycerol-filled, mature fat cells (Cook & Kozak, 1982).

In conclusion, the adipose conversion process should involve at least two separate events which both correspond to the expression of a set of protein markers. This differential expression relates also to a differential regulation by growth hormone of the gene expression of the same markers (Doglio *et al.*, 1986).

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